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(54) Title: PROCEDURE FOR RELIEVING CELL MIXTURES AND TISSUES OF UNWANTED POPULATIONS

(57) Abstract

The present invention relates to a method to relieve cell mixtures and tissues of unwanted populations by means of monoclonal antibodies. Likewise, the subject of the invention is a method to produce photosensibilizing substance-containing conjugates applicable both in vivo and vitro. Another object of the invention is the method for selective elimination of certain types of cells within a living tissue. The above processes are based on the use of monoclonal antibodies conjugated with porphyrine derivatives and laser beam.

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PROCEDURE FOR RELIEVING CELL MIXTURES AND TISSUES OF UNWANTED POPULATIONS

Technical field

The present invention relates to a method to relieve cell mixtures and tissues of unwanted populations by means of monoclonal antibodies. Likewise, the subject of the invention is a method to produce photosensibilizing substance-containing conjugates applicable both in vivo and vitro.

Another object of the invention is the method for selective elimination of certain types of cells within a living tissue.

Background art

Targeted selective elimination of certain cell types from mixed cell populations or from tissues causes problems both in the experimental research work and in the clinical practice. For this purpose the application of carrier-bound /i. e. immunglobuline-bound/ cytotoxic substances was attempted /see for example Keith, A. & al. Cancer Imm. Immunother. /1981/ 12 39-41/. This constitutes the theoretical background of the so called targeting therapy in the course of which monoclonal antibodies are conjugated with strongly cytotoxic substances, like alpha chain of ricin. The selectivity of the method depends solely on the specificity of the carrier molecule. Since it is proven for more and more antibodies, earlier regarded as specific for a single cell type, that the antigen determinant bound by it may be present also on other structures, specificity is considered to be quantitative, rather, than qualitative, characteristics. To eliminate the effect of unwanted binding the combination of locally effective physical effects may offer a solution. This underlines the importance of the normally harmless molecules becoming toxic only by physical influence /e. g. light irradiation/.

Phototherapy is a field of growing importance in medicine. Among the applications of light sources, directed towards specific targets, procedures using laser beams have received special attention. Beside the large power density, the unique physical properties /coherency, polarisation/ of the laser beam add greatly to the elicited biological effect.

In the course of testing the biological effects of a low power He-Ne laser on in vitro cell cultures we could demonstrate a definite energy dependent response. At a given irradiation energy level biological activation, while at a threshold level higher, than this cell disruption could be observed. This phenomenon is described in the Kisérletes Orvostudomány /1984/ 36 96 and the Studia Biophys /1985/ 105 144.

By applying different photosensibilizing molecules the energy threshold values of biological activation and cell disruption can be influenced.

It is known from the prior art that phorphyrine derivates can be well activated at the wavelength range of visible light thereby they are extensively used as photosensibilisers /see for example Br. J. Cancer /1979/ 39 398, Photochem. Photobiophys. /1988/, 10 53-59 and Cancer Res. /1981/ 41 401.

Disclosure of Invention

The present invention is based on the observation that contacting the surface of various cells with photosensibilising substances /and among these with the by itself non toxic and by our He-Ne laser directly non-activable HPD molecule/, followed by illumination with laser beam, results in cell disruption at already such an energy level where non-sensibilised cells would not be destroyed.

To attain this goal porphyrine derivatives, preferably hematoporphyrine hydrochloride is covalently coupled to molecules /monoclonal antibodies/ capable of selective binding to structures on the cell surface.

Coupling is performed by incubation in aqueous solution of the monoclonal antibody protein with the porphyrine derivative, preferably with hematoporphyrine hydrochloride.

Illumination of these hematoporphyrine derivatives by visible light results in a wave length dependent free radical induction process that causes damage to the biological structures. Since the absorption maximum of hematoporphyrine is around 405 nm, after illumination at this wavelength a large amount of free radicals is produced and their damaging effect on living cells cannot be controlled. In contrast to this, at the wavelength range around 630 nm the effect is greatly reduced since the energy of light is absorbed by the biological structure itself rather, than by the hematoporphyrine molecule and, by virtue of electron transfer processes, a much more limited effect is brought about, amenable to more efficient regulation. The role of photosensibilizer molecules in this process is to bring forth electron transfer.

The application of He-Ne laser with 632.8 nm wavelength of emitted light allows for the relatively short term, concentrated photo-energy flux sufficient for initiating the hematoporphyrine regulated electron-transfer processes but not leading directly to free radical generation.

The subject of the present invention is, therefore, a process for the production of photosensibilizing conjugates applicable for both in vivo and in vitro in the course of which, for example a-PNAr-I, a-PNAr-II, a-PNAr-III, a-WGA, a-T3, anti-AFP, a-hCG and anti-H antigens are incubated with certain porphyrine derivatives in aqueous medium.

The characteristics of the above mentioned target-specific monoclonal antibodies are as follows:

a-PNAr-I

Monoclonal antibody /IgG1/ reacting with pea-nut agglutinin antigen /PNA/ binding receptor isolated from the ephithelial cells of the gastric mucosa by lectin affinity chromatography. It reacts with the Golgi region of the cells in the normal gastric mucosa, as well as with

surface and cytoplasmic structures on gastric cancerous cells. The latters specifically bound the monoclonal antibody also in their metastates.

a-PNAr-II

Monoclonal antibody /IgG1/ against PNA binding receptor isolated from milk fat-body membrane by lectin affinity chromatography. The antibody reacts with the basal lamina of the efferent duct of normal breast, as well as with the surface and cytoplasmic structures of breast cancer cells both in primary tumor and in metastates.

a-PNAr-III

Monoclonal antibody /IgG/ against PNA receptor isolated from the mucus of ovarian cyst by lectin affinity chromatography. The antibody reacts well with various ovarian cancer types inboth the primary tumor and metastates.

a-WGA

Monoclonal antibody /IgG/ reacting with germ agglutinin antigen /WGA/ lectin. Following incubation with WGA, cells possessing WGA binding receptors on their surface can be sensitized by it.

anti-T3

Antibodies reacting with T lymphocytes. They can be used at bone-marrow transplantations to remove T cells.

anti-H-antigen

Monoclonal antibody reacting with the surface antigen of the Y chromosome-carrying bull sperm used for insemination.

anti-AFP

Monoclonal antibody reacting with alpha-foetoprotein producing tumors.

anti-hCG

Monoclonal antibody reacting with tumors producing human choriogonadotropine.

Brief Description of Drawings

Fig. 1 demonstrates the test result with a conjugate obtained according to the invention.

Fig. 2 shows the results of example 1 carried out with a-OVA and a-WGA cells.

Best Mode of Carrying out the Invention

Figure 1 demonstrates the test results with a conjugate produced via the aforementioned method. The a-PNAr-I monoclonal antibody, the one raised against the pea-nut agglutinine antigen-binding receptor that is present on the surface of normal cells of the gastric mucosa, as well as on the neoplastically transformed forms, was conjugated with hematoporphyrine followed by freeze-drying. The freeze-dried monoclonal antibody-HP can be stored indefinitely if desiccated. Before use, the conjugate is dissolved in sterile distilled water and the determination of the specific antibody activity and photosensibilizer binding is performed afterwards.

The conjugate prepared this way is suitable for the purposes of in vitro and in vivo laser photo-immunotargeting.

In the in vitro process, incubation of the cell mixtures /1-1.5 hours/ is followed by washing with the culture medium and, afterwards, the mixtures are illuminated by laser at 14 J/cm^2 irradiation energy at practically above 600 nm, preferably at 632 nm wavelength /He-Ne laser/.

Based on our experiments the optimal concentration of the conjugate was 5 micrograms/ml HP.

The present invention, therefore, provides a method to remove unwanted populations from cell mixtures and tissues.

It occurs several times that separation of certain cell populations /e. g. specific monoclonal antibody-producing hybridomes/ is very difficult, moreover, even if the separation is complete the yield of pure cells is very low.

In accordance with the present invention the above problem can be solved by contacting the mixture of the desired and contaminating cells with the conjugate of certain porphyrine derivatives coupled to the contaminating cell/s/-specific monoclonal antibody and submitting the mixture to laser irradiation.

By the above invention the ratio of the desired cells can be markedly increased.

The possibilities provided by the invention can be preferably exploited in the production of monoclonal antibodies specific for small molecular weight substances. In these instances the antigen is conjugated for immunization usually to some high molecular weight protein like thyreoglobuline, bovine serum albumin, key-hole limpit hemocyanine, etc. After cell fusion, therefore, a number of hybridomes occur producing antibody that reacts with the antigen determinant of the carrier protein. Their elimination considerably improves the chances of growing and isolation of the clones.

As it has already been mentioned the procedure, according to the invention, is applicable not exclusively for the production of hybridome cells but, by selecting appropriate monoclonal antibodies, suitable also for the elimination of T-cells in the course of bone-merrow transplantations, as well as to decrease the concentration of Y-chromosome carrying bull sperm used for insemination.

According to the invention, also immuno-targeting method is provided, wnerein living tissue containing non-desired type/s/ of cells is treated with monoclonal antibodies conjugated with porphyrine derivatives and subsequently irradiated with laser beam of a wavelength higher than that activating the said porphyrine derivative. The method is preferably

carried out with He-Ne laser at a wavelength of higher than 600 nm, preferably 630 nm.

Under in vivo circumstances, selective disruption of various cells without impairing their environment is of utmost importance. This hypothesis is substantiated by the following experimental findings.

Human gastric cancer cells, as well as mouse hybridome cells were transplanted dorsally into nude /hairless, thymus deficient/ mice by subcutane injection. As soon as the tumor size reached 0.5 cm, mice were treated with conjugate corresponding to 5-10 mg/kg body weight HP. As conjugates, a-PNAr-I monoclonal antibody conjugate for the human tumor bearing mice and WGA-HP complex for mice transplanted with a-WGA hybridome, were used. After 24 hours the tumors growing on the back of mice were irradiated through the skin by 14 J/cm^2 energy.

For both models HP conjugate by itself did not influence the growth of the tumor. Likewise, laser irradiation by itself has not changed tumor proliferation. However, on the simultaneous application of both stimuli the tumor was destructed very rapidly, within one day. Following the above treatment, the tumor has completely disappeared leaving behind only callus. The process was monitored also by histological follow-up.

The results lend credit to the applicability of in vitro phototargeting processes. Their role extends beyond the human health care also to the veterinary field, e. g. to make animals barren.

The procedure, according to the present invention, is described by the following examples.

Example 1

A mixture of hybridome cells is prepared. 1×10^1 WGA cells are mixed at 1:1 ratio with a -OVA hybridome cells. The former cell line, producing monoclonal immune gamma globuline specifically reacting with wheat germ agglutinine lectin /WGA/, was establisned in our laboratory in 1984, while the latter cell line was developed by Bötcher et al.

in 1978. Conjugate HP-OVA, corresponding to 5 micrograms/ml of HP was added to the RPMI-1640 medium and the mixture was incubated for one hour in a CO_2 thermostate. The cells were then centrifuged and washed three times in conjugate free RPMI-1640 medium. The cell mixture was irradiated by He-Ne laser at 14 J/cm^2 energy. Uniform irradiation could be achieved by a method developed by us earlier by reducing the amount of the culturing medium to the diameter of the laser beam. Before and after irradiation specific antibody production was determined by ELISA and from this the ratio of cell population, producing an individual immunglobuline, could be assessed. The results are shown in Figure 2.

As we can see from the Figure 2, the amount of antibody produced by the anti-OVA cell population rapidly decreased after irradiation and remained at the detection limit for a week. Since we performed a single irradiation, surviving a-OVA cells still remained in the cell mixture, although their number was very low. However, measurable antibody production /detectable not earlier, than one week, corresponding to 10 duplications for hybridome cells equal to 10-100 surviving cells for 2×10^5 total cell count.

Based on the above results, one single irradiation results in orders of magnitude higher efficiency as compared to the already known cell separation processes, like cell sorter.

Example 2

Preparation of the conjugate

20.0 mg hematoporphyrine dihydrochloride dissolved by means of 0.8 ml N,N-dimethyl formamide in 1.25 ml distilled water, is mixed with 20.0 mg 1-ethyl-3-/3-dimethyl-aminopropyl/ carbodiimide hydrochloride /EDCL/ dissolved in 0.6 ml of water. After 30 min 15.0 mg antibody protein, dissolved in 5.0 ml distilled water is added to the solution and the mixture is incubated at room temperature for 5 hours

while the pH is continuously maintained between 6-7. Thereafter 50 microliters of monoethanol-amine is given and the solution is left at room temperature overnight. Following this step the solution is dialysed against 0.0001 M phosphate buffer for 4 days changing the buffer 3 times daily and finally against FBS overnight /pH is kept always at 7.4/. The resulting solution is filtered through Sephadex 625. Following the determination of the total protein content HP binding can be assessed photometrically at 390 nm wavelength by a calibration curve. The activity of the specific antibody can be determined by immunserology /e. g. by ELISA/.

Claims

1. A method for the preparation of conjugates suitable for laser immunotargeting, comprising incubation of a monoclonal antibody with porphyrine derivative in aqueous medium.

2. A method according to claim 1, wherein as monoclonal antibodies a-PNAr-I, a-PNAr-II, a-PNAr-III, a-AFP, a-HCG and a-WGA monoclonal antibodies are applied.

3. A method according to claim 1, wherein as monoclonal antibodies a-T3, anti-AFP and anti-H antigen are applied.

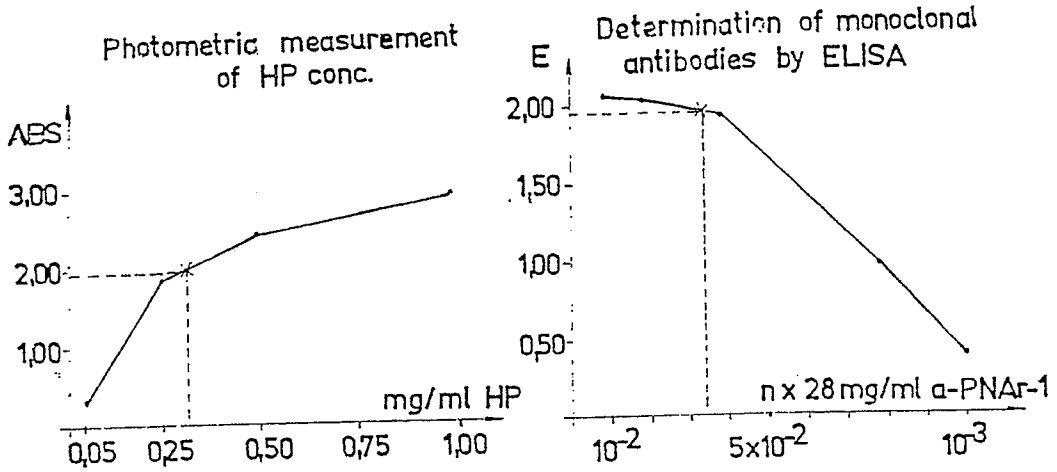
4. A method according to claim 1, wherein as porphyrine derivative hematoporphyrine is applied.

5. A method to remove unwanted population from cell mixtures and tissues, wherein to the mixture of the desired and unwanted cells or tissues the conjugate formed between the antibody reacting with the contaminating cell or cells or tissues and porphyrine derivatives is added and this mixture is subjected to irradiation.

6. A method according to claim 5, wherein laser beam at around 630 nm wavelength is applied.

7. Immunotargeting method, characterized in that living tissue containing non-desired type/s/ of cells is treated with monoclonal antibodies conjugated with porphyrine derivatives, the said monoclonal antibodies being specific for the non-desired cells, and subsequently irradiated with laser beam of a wavelength higher than that activating the said porphyrine derivative.

8. The method according to claim 7, wherein laser beam at around 630 nm wavelength is applied.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mg/ml HP
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0,50
0,75
1,00
Determination of monoclonal antibodies by ELISA
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10-2
5x10-2
10-3

Fig.1

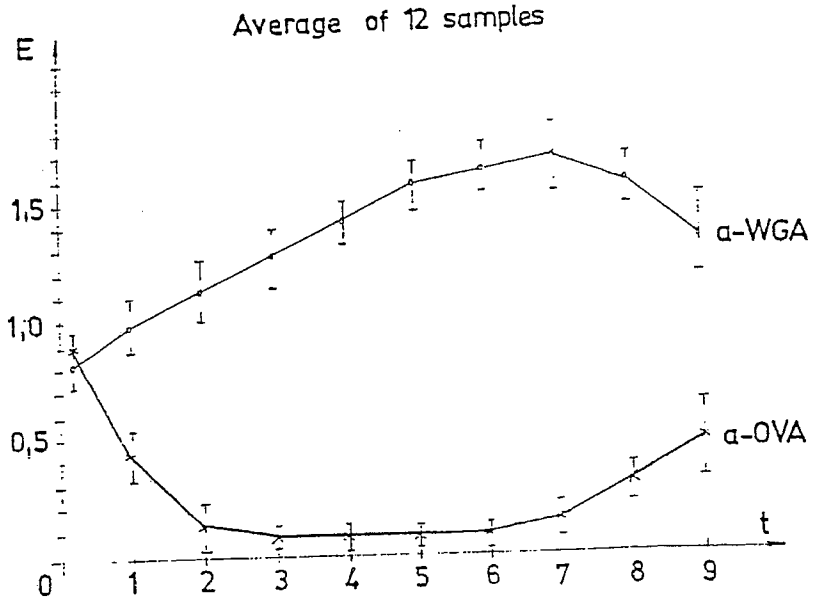
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Fig. 2

INTERNATIONAL SEARCH REPORT

International Application No PCT/HU 88/00057

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[4]: G 01 N 33/58,33/532,33/533,33/577; C 12 N 13/00,5/00; C 12 P 21/00

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
Int.Cl.[4]	G 01 N 33/58,33/532,33/533,33/577; C 12 N 13/00, 5/00; C 12 P 21/00

Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
A	GB, A, 2 063 469 (FISHER SCIENTIFIC COMPANY) 03 June 1981 (03.06.81), see abstract; example VI B.	(1,4)
A	WO, A1, 86/02 734 (HYPERION CATALYSIS INTERNATIONAL, INC.) 09 May 1986 (09.05.86), see abstract; claims 1,16,18.	(1,2)
A	EP, A1, 0 218 352 (NIPPON ZEON CO. LTD.) 15 April 1987 (15.04.87), see abstract.	(5,7)

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IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
25 October 1988 (25.10.88)	10 November 1988 (10.11.88)
International Searching Authority	**Signature of Authorized Officer**
AUSTRIAN PATENT OFFICE	Pippan

Anhang zum internationalen Recherchenbericht über die internationale Patentanmeldung Nr.

Annex to the International Search Report on International Patent Application No. PCT/HU 88/00057

Annexe au rapport de recherche internationale relatif à la demande de brevet international n°.

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Im Recherchenbericht angeführtes Patentdokument Patent document cited in search report Document de brevet cité dans le rapport de recherche	Datum der Veröffentlichung Publication date Date de publication	Mitglied(er) der Patentfamilie Patent family member(s) Membre(s) de la famille de brevets	Datum der Veröffentlichung Publication date Date de publication
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